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G Protein-coupled Receptor Kinase 6A Phosphorylates the Na^+/H^+ Exchanger Regulatory Factor via a PDZ Domain-mediated Interaction*

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The Na^+/H^+ exchanger regulatory factor (NHERF) is constitutively phosphorylated in cells, but the site(s) of this phosphorylation and the kinase(s) responsible for it have not been identified. We show here that the primary site of constitutive NHERF phosphorylation in human embryonic kidney 293 (HEK-293) cells is Ser^{289}, and that the stoichiometry of phosphorylation is near 1 mol/mol. NHERF contains two PDZ domains that recognize the sequence S/T-*X*-L at the carboxyl terminus of target proteins, and thus we examined the possibility that kinases containing this motif might associate with and phosphorylate NHERF. Overlay experiments and co-immunoprecipitation studies revealed that NHERF binds with high affinity to a splice variant of the G protein-coupled receptor kinase 6, GRK6A, which terminates in the motif T-R-L. NHERF does not associate with GRK6B or GRK6C, alternatively spliced variants that differ from GRK6A at their extreme carboxyl termini. GRK6A phosphorylates NHERF efficiently on Ser^{289} *in vitro*, whereas GRK6B, GRK6C, and GRK2 do not. Furthermore, the endogenous "NHERF kinase" activity in HEK-293 cell lysates is sensitive to treatments that alter the activity of GRK6A. These data suggest that GRK6A phosphorylates NHERF via a PDZ domain-mediated interaction and that GRK6A is the kinase in HEK-293 cells responsible for the constitutive phosphorylation of NHERF.

Cellular pH and sodium concentration are regulated in most cell types by a variety of Na^+/H^+ exchanger (NHE)[1] isoforms. The activity of these exchangers in cells can be dramatically affected by hormones or neurotransmitters, many of which regulate NHE activity by altering intracellular levels of cyclic AMP (cAMP) (1–3). The Na^+/H^+ exchanger regulatory factor (NHERF) was originally identified as a 50-kDa protein cofactor required for cAMP-dependent protein kinase (PKA)-mediated inhibition of NHE3 (4, 5). There is evidence that PKA facilitates NHERF-mediated inhibition of NHE3 through both phosphorylation of the exchanger (6) and of NHERF (7). *In vitro* studies have identified a cluster of serines in NHERF as the key region of regulatory phosphorylation by PKA (7), but the phosphorylation of these sites in cells has not been examined.

NHERF contains two PDZ (for PSD-95, Discs-large, and ZO-1 homology) domains, which are protein-protein interaction modules that mediate binding to specific carboxyl-terminal motifs on target proteins (8). The first ligand identified for the NHERF PDZ domains was the tail of the β_2-adrenergic receptor (β_2AR) (9). The interaction of NHERF with the β_2AR is of high affinity (K_D = 18 nM) (10) and plays a role in β_2-adrenergic regulation of NHE3 activity (9). NHERF recognizes the motif S/T-*X*-L at the carboxyl terminus of the β_2AR (10) and also binds with high affinity to other proteins that terminate in this motif, such as the cystic fibrosis transmembrane conductance regulator (10–12).

Some proteins that have potential NHERF binding motifs are protein kinases, raising the possibility that cellular NHERF may be constitutively associated with one or more kinases. It has recently been observed that NHERF is phosphorylated under basal conditions in various cell lines (7, 13), but the identity of the NHERF kinase responsible for this phosphorylation is unknown. In the present study, we examined the phosphorylation of cellular NHERF under basal conditions as well as following various stimulatory treatments. We also tested the idea that NHERF might associate with a protein kinase through a PDZ domain-mediated interaction, and that this interaction might explain the constitutive phosphorylation of cellular NHERF.

EXPERIMENTAL PROCEDURES

Culture and Transfection of Human Embryonic Kidney 293 (HEK-293) Cells—HEK-293 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in 75% Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (all from Life Technologies, Inc.) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. HEK-293 cells were subcultured every week after becoming confluent using 0.25% trypsin with 1 mM EDTA (Life Technologies, Inc.).

Metabolic Labeling and Immunoprecipitation of Epitope-tagged NHERF—HEK-293 cells were plated in 100-mm dishes and grown to approximately 80% confluence. Cells were then transfected with 1–2 μg of HA-NHERF cDNA using the calcium-phosphate method, as described previously (9). Forty-eight hours following transfection, the medium was replaced with 6 ml of phosphate-free Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 0.4 mCi of ^{32}P (NEN Life Science Products). After 90 min, cells were incubated with the agents to be tested or their vehicles at the indicated concentrations and for the indicated times. Following treatment, cells were

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[1] The abbreviations used are: NHE, Na^+/H^+ exchanger; NHERF, Na^+/H^+ exchanger regulatory factor; PKA, cyclic AMP-dependent protein kinase; β_2AR, β_2-adrenergic receptor; HEK-293, human embryonic kidney 293; PAGE, polyacrylamide gel electrophoresis; GRK, G protein-coupled receptor kinase; sgk, serum and glucocorticoid regulated kinase; CaMKII, calcium/calmodulin-dependent protein kinase II; PCR, polymerase chain reaction; HA, hemagglutinin; GST, glutathione *S*-transferase; Ab, antibody.

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washed three times with 6 ml of Dulbecco's phosphate-buffered saline and then were scraped into 1 ml of ice-cold lysis buffer containing 150 mM NaCl, 50 mM Tris, pH 8.0, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 2 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 50 nM calyculin, 1 mM benzamide, and 100 μM phenylmethanesulfonyl fluoride. The lysate was transferred to a 1.5-ml microcentrifuge tube and then rocked for 30 min at 4 °C. Insoluble material was removed by centrifugation at 10,000 $\times$ *g* for 4 min. One milliliter of supernatant was transferred to a 1.5-ml microcentrifuge tube, and 6 μg of 12CA5 monoclonal antibody (Roche Molecular Biochemicals) was added. After rocking at 4 °C for 1 h, 60 μl of protein A plus protein G-agarose (Calbiochem, La Jolla, CA) was added and the samples rocked at 4 °C for 1 h. The protein A-agarose was then washed three times in lysis buffer. SDS-sample buffer (100 μl) was added to the pellet and heated to 85 °C for 10 min. Proteins were separated on a 12% SDS-polyacrylamide gel. After drying the gels, phosphorylated proteins were detected by autoradiography. Determination of the stoichiometry of NHERF phosphorylation was performed as described previously in studies examining the stoichiometry of β_1-adrenergic receptor phosphorylation (14).

Western Blotting—To verify expression and immunoprecipitation of NHERF, precipitated samples and cell lysates were run on SDS-PAGE gels, as described in the preceding section, and then blotted onto pure nitrocellulose (Novex) for 40 min at 12 V. The blots were blocked in blot buffer (2% milk, 0.1% Tween 20, 10 mM HEPES, pH 7.4, 50 mM NaCl) for 20 min and then incubated with either anti-NHERF (9) or 12CA5 antibody for 1 h at room temperature. The blots were then washed three times for 5 min each in 10 ml of blot buffer and incubated for 1 h at room temperature with either anti-rabbit or anti-mouse horseradish peroxidase-coupled secondary antibody (Amersham Pharmacia Biotech) at a dilution of 1:2000. Following this, the blots were again washed three times in 10 ml of blot buffer, once with phosphate-buffered saline, and visualized via chemiluminescence with an ECL detection kit (Amersham Pharmacia Biotech) and Kodak X-Omat film.

Creation of NHERF Mutants—Four mutant versions of NHERF were prepared for this study: S287A, S289A, S290A, and K158A,K159A. These mutants were created by PCR amplification from the native rabbit NHERF cDNA using mutant sequence oligonucleotides; all mutations were confirmed via sequencing with an ABI377 automated sequencer. The mutants were inserted into both the HA-pBK-CMV vector (9) for expression in mammalian cells and pET-30A for fusion protein expression in *Escherichia coli*.

NHERF Overlays—Hexahistidine- and S-tagged NHERF, NHERF domain 1, and NHERF domain 2 fusion proteins ("domain 1" = residues 1–151 of full-length rabbit NHERF; "domain 2" = residues 152–358) were expressed and purified as described previously (7). GRK6A, GRK6B, and β_2 receptor carboxyl-terminal tails were expressed as GST fusion proteins by PCR amplification from native cDNAs and insertion of the PCR products into the pGEX-2T vector (Amersham Pharmacia Biotech); the sequences of all constructs were confirmed via sequencing with an ABI377 automated sequencer. The GST fusion proteins (5 μg/lane) were run on 4–20% SDS-PAGE gels (Novex), blotted as described above and overlaid with the appropriate fusion protein in blot buffer for 1 h at room temperature. The blots were then washed three times with blot buffer, incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-S-tag Ab (Novagen) in blot buffer, washed three more times with blot buffer, and visualized via chemiluminescence. To perform saturation binding curves, equal amounts of a given fusion protein were loaded into multiple lanes, and the resultant blots were cut into one-lane strips. The strips were incubated with increasing concentrations of NHERF domain 1 fusion protein, and the amount of NHERF binding for each was quantified via scanning and analysis with the program IPLab (Scientific Analytics Corp.).

Kinase Assays—Full-length mouse GRK6A, GRK6B, and GRK6C cDNAs were amplified from libraries and subcloned into the pBK-CMV vector for the expression studies. Hexahistidine-tagged GRK6 fusion proteins were expressed via baculovirus in Sf9 cells and purified using Ni-NTA chromatography with extensive washing to remove imidazole. GRK2 was expressed via baculovirus in Sf9 cells and GRK2 was purified as described previously (15). The purified proteins were examined for their ability to phosphorylate wild-type or mutant versions of NHERF *in vitro*. Kinase assays were carried out at room temperature (24 °C) in a total volume of 30 μl, with 20 mM Tris, pH 7.4, 10 mM $MgCl_2$, 2 mM EDTA, and 1 mM dithiothreitol. For experiments comparing the efficiencies of different GRKs to phosphorylate NHERF, the kinases were normalized for their ability to phosphorylate the GRK substrate peptide RRREEEEESAAA (16). Typically, kinases were added to 2 μg of substrate, unless otherwise indicated; for the kinetic analyses, the amount of substrate added was varied. Reactions were initiated via the addition of ATP to a final total concentration of 60 μM, with 10 μCi of [γ-^{32}P]ATP (NEN Life Science Products) per reaction. Reactions were allowed to proceed for 10 min before being stopped with sample buffer. For phosphorylation *in vitro* with calcium/calmodulin-dependent protein kinase II (kindly supplied by T. R. Soderling, Vollum Institute, Portland, OR), 5 mM $CaCl_2$and 1 μM calmodulin were included in the phosphorylation reactions. Phosphorylated samples were run on 4–20% SDS-PAGE gels, fixed, dried, and exposed to film. The extent of phosphorylation was quantitated by scanning of films and densitometric analysis of bands.

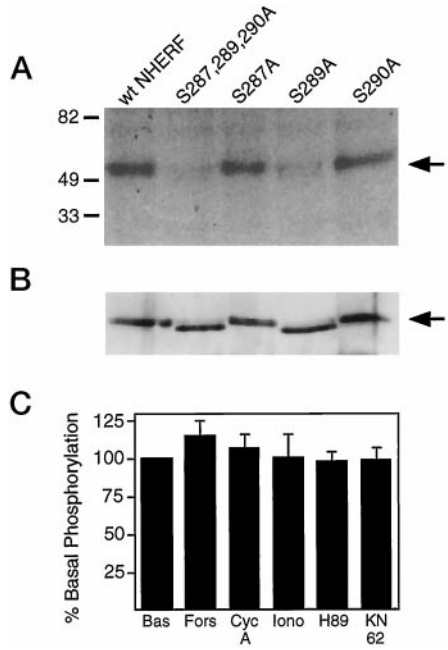

FIG. 1. **NHERF is constitutively phosphorylated on serine 289 in HEK-293 cells.** *A*, mutation of Ser^{289} reduces NHERF phosphorylation. The ^{32}P content of wild-type NHERF immunoprecipitated from ^{32}P-labeled 293 cells is compared in this autoradiogram to the ^{32}P content of four different mutant versions of NHERF: a mutant with Ser^{287}, Ser^{289}, and Ser^{290} all changed to alanine (7) (*lane 2*), or mutants with these three serines individually changed to alanine (*lanes 3–5*). Only mutation of Ser^{289} reduces NHERF phosphorylation relative to wild-type. Molecular mass markers (in kDa) are shown at the *left* side of the panel; the phosphorylated NHERF band is indicated by the *arrow*. *B*, the majority of NHERF in HEK-293 cells is phosphorylated on Ser^{289}. This anti-NHERF Western blot, to detect HA-tagged NHERF immunoprecipitated from HEK-293 cells (indicated by the *arrow*), demonstrates that mutation of Ser^{289} shifts the SDS-PAGE mobility of NHERF. *C*, treatment with agents that activate or inhibit PKA or CaMKII have little effect on NHERF phosphorylation in HEK-293 cells. ^{32}P-Labeled HEK-293 cells were stimulated with either 10 μM forskolin (*Fors*) for 10 min, 50 μM 8-bromo-cyclic AMP (*Cyc A*) for 10 min, 1 μM ionomycin (*Iono*) for 10 min, 1 μM H89 for 2 h, or 1 μM KN-62 for 2 h. The NHERF from these cells was then immunoprecipitated, and the ^{32}P content was quantitated and expressed as a percentage of basal NHERF phosphorylation (*Bas*). Each *bar* and *error bar* represents the mean ± S.E. for 3–8 independent determinations.

RESULTS

It has been shown previously that NHERF inhibition of NHE3 *in vitro* can be enhanced by phosphorylation of NHERF within a cluster of three serines, Ser^{287}, Ser^{289}, and Ser^{290}, located toward the carboxyl-terminal end of the NHERF protein (7). In order to see if one or more of these sites is responsible for the previously observed constitutive phosphorylation of NHERF in cells (7, 13), point mutants of NHERF were prepared with these three serines individually mutated to alanine. These mutants, as well as a triple mutant with all three serines changed to alanine, were separately transfected into HEK-293 cells and their levels of phosphorylation were as-

sessed in ^{32}P metabolic labeling studies. As shown in Fig. 1*A*, wild-type NHERF is constitutively phosphorylated, while the triple alanine mutant is reduced in phosphorylation by approximately 75% relative to wild-type NHERF. The S287A and S290A point mutants exhibit levels of constitutive phosphorylation identical to wild-type NHERF, but the S289A point mutant is reduced in constitutive phosphorylation by approximately 75%. These results indicate that the primary site of constitutive phosphorylation in NHERF expressed in HEK-293 cells is Ser^{289}.

We next examined the stoichiometry of this constitutive phosphorylation. The stoichiometry was calculated at 0.74 ± 0.06 mol/mol ($n = 3$), a value close to 1 and thus consistent with NHERF being constitutively phosphorylated on one primary site. The Western blot data shown in Fig. 1*B* are also consistent with this idea. It has previously been observed that Western blot staining of native tissues with anti-NHERF antibodies yields several closely spaced immunoreactive bands that can be collapsed into a single band by treatment with alkaline phosphatase (17), suggesting that NHERF is phosphorylated in native tissues and that this phosphorylation retards the mobility of the NHERF protein on SDS-PAGE. As seen in Fig. 1*B*, the triple mutant and S289A mutant forms of NHERF exhibit more rapid mobility on SDS-PAGE gels, suggesting that phosphorylation on Ser^{289} retards the SDS-PAGE mobility of NHERF. Since most of the NHERF in the triple mutant and S289A mutant samples is shifted to the apparently smaller size, it is likely that most of the wild-type NHERF expressed in these cells is phosphorylated on Ser^{289}.

To see if the NHERF phosphorylation on Ser^{289} might be due to activity of PKA or CaMKII, two kinases previously shown to inhibit NHE3 activity *in vitro* (4, 7, 18, 19), we performed ^{32}P metabolic labeling studies on wild-type NHERF-transfected HEK-293 cells treated with a variety of activators or inhibitors of these two kinases. As shown in Fig. 1*C*, none of the PKA- or CaMKII-targeted treatments had a significant effect on the extent of NHERF phosphorylation. Treatment with forskolin led to a small (~15%) but statistically insignificant increase in NHERF phosphorylation, as observed previously (7), while treatment with 8-bromo-cAMP, the calcium ionophore ionomycin, the PKA inhibitor H89 or the CaMKII inhibitor KN-62 led to no significant changes in the phosphorylation state of NHERF.

Since most of the NHERF in the HEK-293 cells was phosphorylated even in the absence of any stimulation, we considered the possibility that NHERF might be associated in these cells with a constitutively active kinase that was able to keep NHERF phosphorylated on Ser^{289} at all times. NHERF has two PDZ domains, which could potentially allow association of NHERF with one or more kinases. The carboxyl-terminal tail motif preferred for binding by the NHERF PDZ domains is S/T-*X*-L (10, 11). We examined two kinases on the data base that terminate in such a motif: the serum and glucocorticoid regulated kinase (sgk) (20) and the G protein-coupled receptor kinase 6 (GRK6) (21, 22). sgk terminates in D-S-F-L, a carboxyl terminus very similar to the D-S-L-L C terminus of the β_2-adrenergic receptor, while GRK6A, a major splice variant of GRK6, terminates in P-T-R-L.

The potential interaction of NHERF and sgk was assessed in overlay studies and in co-immunoprecipitation experiments. In no case did we observe binding of NHERF to either sgk or the sgk tail, nor did we observe any phosphorylation of NHERF by sgk (data not shown). These studies reveal that, while a COOH-terminal S/T-*X*-L motif may be necessary for high affinity binding to the first NHERF PDZ domain, it is not sufficient. There must be other structural determinants in the sgk tail region that prevent it from associating with the NHERF PDZ domains.

GRK6 exists as three alternatively spliced variants (22),[2] as shown in Fig. 2*A*. Only the first of these variants (GRK6A) possesses the T-R-L carboxyl terminus that conforms to the consensus motif for NHERF binding. We prepared GST fusion proteins corresponding to the carboxyl termini of GRK6A and GRK6B, the most abundant of the GRK6 isoforms, and compared the binding of NHERF to these two samples (Fig. 2*B*). Full-length NHERF binds specifically to the carboxyl terminus of GRK6A but not to the GRK6B carboxyl terminus nor to control GST. The overlay shown in Fig. 2*C* reveals that both of the NHERF PDZ domains bind the GRK6A carboxyl terminus, although the first PDZ domain binds much better than the second. Both PDZ domains bind about as well to the GRK6A carboxyl terminus as they do to the carboxyl terminus of the β_2-adrenergic receptor. Indeed, saturation binding studies (Fig. 2*D*) revealed that NHERF domain 1 binds to the GRK6A carboxyl terminus with an affinity (K_D = 31 nM) similar to that previously estimated for NHERF binding to the β_2AR carboxyl terminus (K_D = 18 nM) (10). Co-immunoprecipitation studies were performed in order to examine whether or not NHERF could bind to full-length GRK6A in a cellular context; as shown in Fig. 2*E*, GRK6A but neither GRK6B nor GRK6C can be co-immunoprecipitated with NHERF from HEK-293 cells.

We next examined whether GRK6A can phosphorylate NHERF. As the experiment shown in Fig. 3*A* illustrates, purified GRK6A efficiently phosphorylates NHERF *in vitro*, while purified GRK6B, GRK6C, and GRK2 phosphorylate NHERF either very poorly or not at all. When equal amounts of NHERF and the classical GRK substrate rhodopsin are presented to GRK6A, NHERF is highly preferred as a substrate (Fig. 3*B*, *lanes 1* and *2*). When the same experiment is performed with GRK2, rhodopsin is strongly preferred as a substrate and NHERF is not detectably phosphorylated (Fig. 3*B*, *lanes 3* and *4*).

To characterize the primary site of GRK6A phosphorylation, the NHERF S287A, S289A, and S290A point mutants were expressed as hexahistidine-tagged fusion proteins, purified, and examined alongside wild-type NHERF for their ability to be phosphorylated by GRK6A. As shown in Fig. 3*C*, the S287A and S290A mutants were phosphorylated by GRK6A as efficiently as wild-type NHERF, while the S289A mutant was not detectably phosphorylated. The phosphorylation of NHERF domain 1 *versus* domain 2 was also examined, to see if the presence of the high affinity GRK6A binding site in the first NHERF PDZ domain is required for NHERF phosphorylation by GRK6A; as shown in Fig. 3*D*, NHERF domain 2 was phosphorylated slightly *better* than full-length NHERF (domain 2 phosphorylation = 127 ± 16% of wild-type; $n = 4$), while domain 1 was not detectably phosphorylated. Metabolic labeling experiments were also performed to examine the cellular phosphorylation of transfected full-length NHERF *versus* domain 2; no significant difference in ^{32}P incorporation per unit protein was observed in three independent experiments. These results reveal that NHERF is a substrate for phosphorylation by GRK6A, that the primary site of phosphorylation is Ser^{289}, and that the first NHERF PDZ domain plays little or no role in mediating GRK6A phosphorylation of NHERF.

The observation that removal of the first PDZ domain of NHERF does not reduce phosphorylation by GRK6A suggested that the second PDZ domain of NHERF might be the GRK6A binding site relevant for phosphorylation. To examine this pos-

[2] R. T. Premont, S. Aparacio, H. E. Kendall, J. E. Welch and R. J. Lefkowitz, submitted for publication.

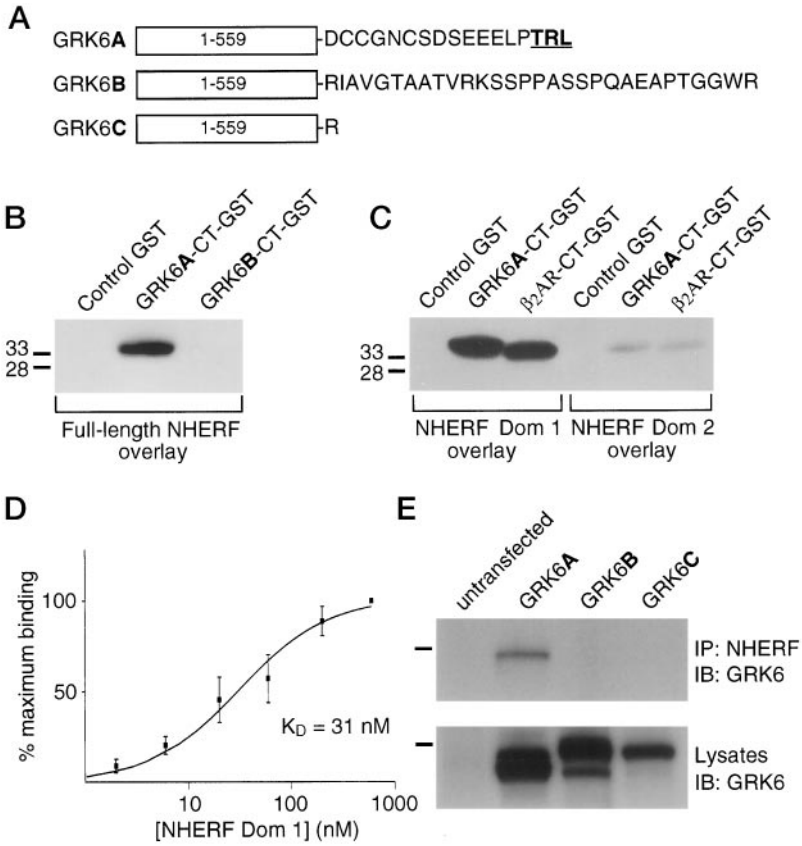

FIG. 2. **NHERF binds to GRK6A.** *A*, schematic diagram of the three known splice variants of mouse GRK6. The first 559 amino acids are identical in each of these proteins, but the tail regions are variable. The consensus carboxyl-terminal NHERF-binding motif (S/T-*X*-L) is shown in *bold* at the end of GRK6A. *B*, full-length NHERF binds to the GRK6A carboxyl terminus but not control GST nor the GRK6B carboxyl terminus. 5 μg of each GST fusion protein was run on an SDS-PAGE gel, blotted, and overlaid with full-length NHERF fusion protein (50 nM). *C*, NHERF domain 1 binds better than NHERF domain 2 to both the GRK6A and β_2AR carboxyl termini. Two sets of 5 μg of GST, GRK6A-CT-GST, and β_2AR-CT-GST were run on a gel, blotted, divided in half, and overlaid with either 50 nM NHERF domain 1 or 50 nM NHERF domain 2. The blot was then reassembled to provide a direct side-by-side comparison of the binding of the two NHERF PDZ domains. The positions of molecular mass markers (in kDa) are indicated at *left*. The blots shown in *B* and *C* are representative of at least three experiments each. *D*, NHERF domain 1 binds with high affinity to the GRK6A carboxyl terminus. Binding of NHERF domain 1 (2–1000 nM) was examined to provide an estimate of the equilibrium binding affinity of the GRK6A/NHERF domain 1 interaction. The *points* and *error bars* represent the mean ± S.E. for three independent experiments. *E*, GRK6A co-immunoprecipitates with NHERF. HA-NHERF was expressed in HEK-293 cells with GRK6A, GRK6B, or GRK6C, and NHERF was immunoprecipitated with a 12CA5 Ab. The precipitates were then probed with an anti-GRK6 antibody (*upper panel*). The *lower panel* shows the anti-GRK6 staining in the cell lysates, revealing that the three splice variants were expressed at comparable levels. The position of the 66-kDa molecular mass marker (bovine serum albumin) is indicated at *left*. This experiment was performed four times with similar results.

sibility, a version of NHERF domain 2 was prepared with a mutation of two amino acids (K158A,K159A) in the second PDZ domain. Crystallographic studies on other PDZ domains have suggested that a positively charged residue at one of these positions is important for interaction with the terminal carboxylate groups of bound peptides (23), and thus mutation of these two lysines to alanines might be expected to reduce the affinity of the second NHERF PDZ domain for binding partners. The positions of lysines 158 and 159 relative to the two NHERF PDZ domains and Ser^{289} are indicated in the schematic diagram of NHERF presented in Fig. 3*E*.

As shown in Fig. 4*A*, binding of 500 nM K158A,K159A NHERF domain 2 to the GRK6A carboxyl terminus was substantially reduced relative to the binding of 500 nM wild-type domain 2 (wild-type = 4.2 ± 0.9-fold higher than K158A,K159A mutant domain 2 binding; $n = 4$). It is not possible with the overlay assay to perform saturation binding studies to estimate the affinity of either the wild-type or mutant domain 2 for the GRK6A carboxyl terminus, since extremely high concentrations of the domain 2 fusion protein (up to 50 μM) would be required to reach saturation binding. The signal-to-noise ratio is very unfavorable under such conditions, as can be seen in the large amount of background staining in the blot overlays shown in Fig. 4*A*.

The phosphorylation of the K158A,K159A mutant domain 2 by GRK6A was examined next. When assayed alongside wild-type domain 2, the K158A,K159A mutant exhibited markedly reduced phosphorylation by GRK6A at low substrate concentrations, while at high substrate concentrations GRK6A-mediated phosphorylation of the K158A,K159A mutant was slightly enhanced (Fig. 4*B*). These findings translate into significantly different K_m and V_{max} values for the mutant NHERF domain 2 relative to the wild-type (wild-type K_m = 0.69 ± 0.13 μM *versus* 2.48 μM ± 0.49 for the mutant; wild-type V_{max} = 10.6 ± 0.5 nmol/min/mg *versus* 14.0 ± 0.9 nmol/min/mg for the mutant). The V_{max}/K_m value for wild-type domain 2 was 2.8-fold higher than that for the K158A,K159A mutant, indicating that the wild-type NHERF domain 2 is a better substrate than the K158A,K159A mutant for phosphorylation by GRK6A.

Since the phosphorylation of NHERF by GRK6A is on the same serine as the constitutive NHERF phosphorylation observed in HEK-293 cells, we wondered if GRK6A might be the kinase responsible for the constitutive cellular NHERF phosphorylation. Most of the reagents known to alter GRK activity

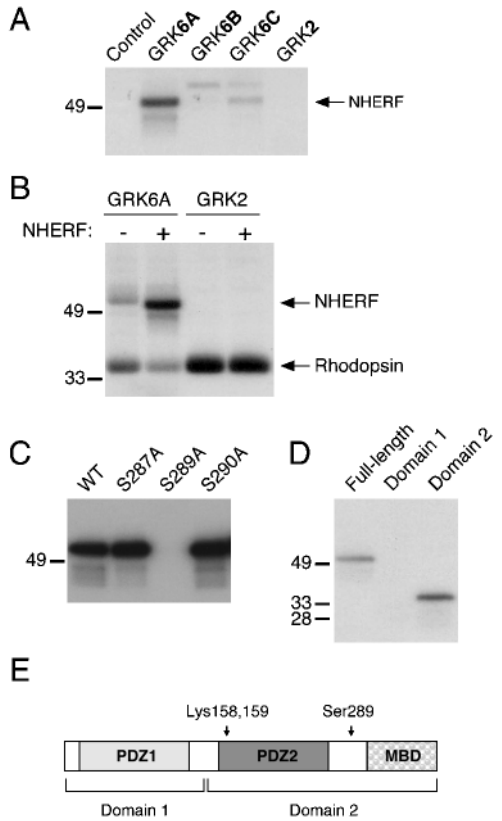

FIG. 3. **GRK6A phosphorylates NHERF.** *A*, GRK6A phosphorylates NHERF efficiently but GRK6B, GRK6C, and GRK2 do not. The four different kinases were normalized for phosphorylation of a GRK substrate peptide and then incubated with 2 μg of NHERF for 10 min. The resultant phosphorylation is shown in this autoradiogram. The slightly phosphorylated band above the NHERF in several of the samples represents autophosphorylated kinase. For the "control" condition, assay conditions were identical except that no exogenous kinase was added. *B*, NHERF is a preferred substrate for GRK6A. Phosphorylation of rhodopsin (2 μg) by GRK6A or GRK2 was assessed in the absence (−) and the presence (+) of NHERF (2 μg). This autoradiogram demonstrates that GRK6A phosphorylates NHERF more efficiently than it phosphorylates rhodopsin, while GRK2 strongly prefers rhodopsin. The slightly phosphorylated band just above NHERF in the first two lanes represents autophosphorylated GRK6A. *C*, GRK6A phosphorylates NHERF on Ser^{289}. 2 μg each of wild-type, S287A, S289A, and S290A NHERF fusion proteins were incubated with GRK6A, and the resultant phosphorylation is shown in this autoradiogram. *D*, domain 1 is not required for NHERF phosphorylation by GRK6A. 5 μg each of full-length, domain 1, and domain 2 NHERF fusion proteins were incubated with GRK6A, and the amount of phosphorylation of each fusion protein is shown in this autoradiogram. This exposure is underdeveloped to reveal the slight increase in domain 2 phosphorylation relative to full-length NHERF. The experiments shown in the *first four panels* of this figure are representative of at least three experiments each, and the *bars* and *numbers* to the *left* of each panel represent molecular mass standards in kDa. *E*, schematic diagram of NHERF. The positions of the two PDZ domains and the MERM (for merlin, ezrin, radixin, and moesin homology)-binding domain (*MBD*) (17, 31) are shown within the context of the domain 1 and domain 2 constructs used in the present study. The relative positions of Lys^{158}, Lys^{159}, and Ser^{289} are indicated by the *arrows*.

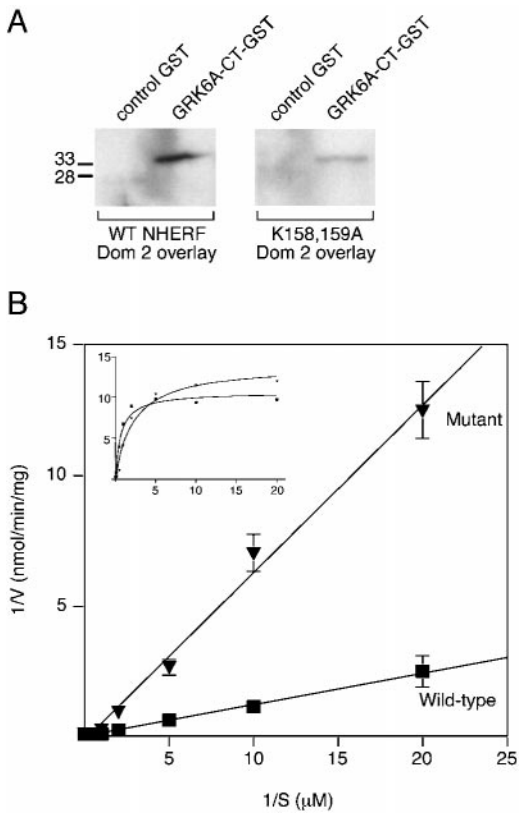

FIG. 4. **GRK6A binds the K158A,K159A NHERF domain 2 mutant with lower affinity and phosphorylates it with lower efficiency.** *A*, The K158A,K159A NHERF mutation impairs binding of the GRK6A carboxyl terminus. 5 μg of control GST and GRK6A-CT-GST were run on a gel, transferred, and overlaid with either 500 nM wild-type NHERF domain 2 or 500 nM K158A,K159A mutant NHERF domain 2. This blot was overexposed to detect the low affinity binding of GRK6A to the K158A,K159A NHERF mutant; thus, the background staining is quite high. The results shown here are representative of four experiments. *B*, the K158A,K159A NHERF domain 2 mutant is phosphorylated with altered kinetics relative to wild-type domain 2. GRK6A phosphorylation of either wild-type (*squares*) or K158A,K159A mutant NHERF domain 2 (*inverted triangles*) was performed at concentrations of substrate ranging from 2 nM to 20 μM. The data are shown as Lineweaver-Burke transformations, while the untransformed data are shown as *V versus* [S] curves in the *inset*; standard errors for the untransformed data are proportional to those for the transformed data, but are not shown to allow greater clarity for the *inset graph*. The *points* and *error bars* in the transformed graph represent the means and standard errors for three independent determinations.

(*e.g.* heparin, anti-GRK antibodies) are not cell-permeable, and thus ^{32}P labeling studies in whole cells cannot easily be performed in the presence of these reagents. We therefore examined the effects of these reagents *in vitro* on phosphorylation of NHERF by both purified GRK6A and the endogenous NHERF kinase activity in HEK-293 cell lysates.

As shown in Fig. 5*A*, heparin potently activated GRK6A kinase activity, both with regard to NHERF phosphorylation (*second bar*) and GRK6A autophosphorylation (data not shown). GRK6A phosphorylation of NHERF was inhibited by anti-GRK4/5/6 monoclonal antibody, but not by anti-GRK2/3 antibody, and was also inhibited by an excess of NHERF domain 1 fusion protein. This latter finding emphasizes that efficient phosphorylation of NHERF by GRK6A is dependent on a PDZ domain-mediated interaction. The phosphorylation of NHERF by GRK6A was also inhibited by high concentrations, but not low concentrations, of the broad spectrum kinase inhibitor staurosporine, consistent with previous findings for GRK2 (14). When the NHERF kinase activity in HEK-293 cell lysates was examined for sensitivity to the treatments shown to affect GRK6A phosphorylation of NHERF, a very similar profile of activation and inhibition was observed (Fig. 5*B*). The NHERF kinase activity in the HEK-293 cell lysates was activated by heparin, unaffected by anti-GRK2/3 antibody or 10 nM staurosporine, and inhibited by anti-GRK4/5/6 antibody, NHERF domain 1, and 10 μM staurosporine. These data suggest that GRK6A is the kinase responsible for the constitutive phosphorylation of NHERF Ser^{289} in these cells.

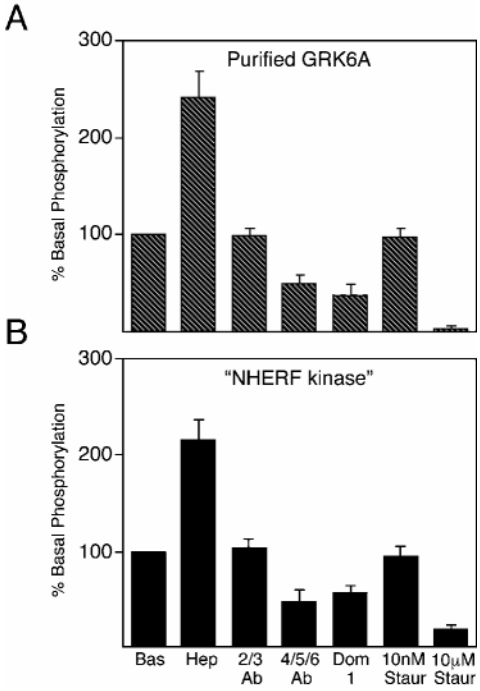

FIG. 5. **The NHERF kinase activity in HEK-293 cell lysates is sensitive to treatments that affect GRK6A.** 2 μg of NHERF was phosphorylated with either purified GRK6A (*A*) or extract from HEK-293 cells (*B*) in the presence of 1 μg/ml heparin (*Hep*), 10 μg/ml anti-GRK2/3 Ab (*2/3 Ab*), 10 μg/ml anti-GRK4/5/6 Ab (*4/5/6 Ab*), 1 μM NHERF domain 1 (*Dom 1*), 10 nM staurosporine (*10 nM Staur*), or 10 μM staurosporine (*10 μM Staur*). The phosphorylation of each sample was quantified and expressed as a percentage of basal phosphorylation (*Bas*) within the same experiment. The *bars* and *error bars* are representative of 3–6 independent determinations for each condition.

DISCUSSION

NHERF was originally identified as the protein co-factor required for regulation of NHE3 by PKA (4, 5), and phosphorylation by PKA has been shown to enhance NHERF inhibition of NHE3 *in vitro* (4, 7). Surprisingly, however, the experiments described here and in two other recent studies (7, 13) demonstrate that modulation of PKA activity has no significant effect on NHERF phosphorylation in cells. This lack of PKA-mediated NHERF phosphorylation may be related to the observation that cellular NHERF is highly phosphorylated even under basal conditions. We therefore sought to characterize the constitutive phosphorylation of NHERF and identify the responsible kinase or kinases. We conclude that almost all of the NHERF in HEK-293 cells is phosphorylated under basal conditions and that the kinase responsible for this phosphorylation is GRK6A.

GRK6A phosphorylates NHERF on Ser^{289}, the primary site of constitutive phosphorylation of NHERF in HEK-293 cells. There is evidence that PKA can also phosphorylate NHERF on Ser^{289}, or an adjacent serine, *in vitro* (7). Ser^{289} furthermore conforms to the ideal R-*X*-*X*-S/T-*X*-E/D motif for CaMKII phosphorylation (24), and we have found that this site is an excellent substrate for CaMKII *in vitro*.[3] However, NHERF Ser^{289} does not seem to be a substrate for PKA or CaMKII in HEK-293 cells, probably due to constitutive phosphorylation of this site by GRK6A. GRK6 is widely expressed; most tissues exhibit a mixture of GRK6A and GRK6B, although the former is predominant in the brain and the latter is predominant elsewhere (22). We have examined NHERF phosphorylation in several cell lines, and found a high level of constitutive phosphorylation in all of them,[4] consistent with the nearly ubiquitous expression of GRK6A.

Is the phosphorylation of NHERF Ser^{289} by GRK6A of physiological relevance? Ser^{289} is located within a cluster of serines that has previously been identified as a key region involved in regulating the ability of NHERF to inhibit NHE3 activity (7). Thus, it is plausible that phosphorylation of NHERF in this region by GRK6A will influence NHERF inhibition of NHE3. The interaction of NHERF and NHE3 is mediated by the region of NHERF encompassing the second PDZ domain and the tail (25), and it is therefore reasonable that phosphorylation of the serine-rich stretch in the center of this region (including Ser^{289}) might affect the physical interaction of NHERF with NHE3.

The phosphorylation of NHERF by GRK6A depends upon the binding of the GRK6A tail to the second NHERF PDZ domain. There are three lines of evidence supporting this contention: (i) NHERF is efficiently phosphorylated *in vitro* by GRK6A but not GRK6B or GRK6C, splice variants that differ only in their extreme carboxyl-terminal regions, (ii) a mutation in the second NHERF PDZ domain that lowers the binding affinity for GRK6A lowers the efficiency with which NHERF is phosphorylated by GRK6A, and (iii) preincubation with a molar excess of NHERF domain 1 reduces the ability of GRK6A to phosphorylate full-length NHERF. This latter effect is presumably due to the fact that NHERF domain 1 binds to the tail of GRK6A with high affinity and prevents the kinase from binding to the second PDZ domain and phosphorylating any full-length NHERF presented to it.

GRK6A binds with extremely high affinity to the first NHERF PDZ domain and with much lower affinity to the second NHERF PDZ domain. Nonetheless, our data indicate that the latter interaction is the relevant one for facilitating NHERF phosphorylation. A mutation that reduces the binding of NHERF domain 2 to the GRK6A tail by approximately 4-fold reduces the efficiency with which GRK6A phosphorylates domain 2 by approximately 3-fold. This difference in the rate of GRK6A phosphorylation of the mutant and wild-type domain 2 fusion proteins is exaggerated at low substrate concentrations, where the affinity of kinase for substrate is a central determinant of the rate of phosphorylation. At high substrate concentrations, where the affinity of kinase for substrate is less important than how rapidly the kinase can move from one substrate to another, the K158A,K159A mutant domain 2 is actually a slightly *better* substrate for GRK6A phosphorylation than wild-type domain 2. This is presumably due to the fact that the lowered affinity of GRK6A for domain 2 translates into a faster off-rate, allowing GRK6A to disengage from substrate more rapidly following phosphorylation.

G protein-coupled receptor kinases were originally identified based on their ability to phosphorylate and desensitize G protein-coupled receptors (26). The first non-receptor GRK substrate identified was tubulin (27–29). NHERF represents the second non-receptor GRK substrate identified, although clearly it is not a substrate for all GRKs but rather is a specific substrate for GRK6A. The requirement for GRK6A binding to NHERF via a PDZ domain-mediated interaction is consistent with the notion that GRKs need to be localized near their substrates in order to phosphorylate (26). Peptide phosphorylation experiments have previously revealed that GRK6 prefers basic residues to the amino-terminal side of phosphorylation sites (30), but no phosphorylation sites within GRK6 substrates have been mapped. The finding made here that Ser^{289} in NHERF is an excellent substrate for GRK6A phosphorylation represents the first defined phosphorylation site

[3] R. A. Hall and R. J. Lefkowitz, unpublished observations.

[4] R. S. Spurney, and R. J. Lefkowitz, unpublished observations.

within a GRK6 substrate, and it is of interest that this site conforms to the R-*X*-*X*-S/T motif proposed to be ideal for GRK6 phosphorylation (30).

In conclusion, we have identified Ser^{289} as the major site of constitutive phosphorylation of NHERF expressed in HEK-293 cells. We have also shown that NHERF binds to GRK6A via a PDZ domain-mediated interaction, that GRK6A phosphorylates NHERF on Ser^{289}, and that the constitutive NHERF kinase activity in HEK-293 cell lysates has many properties in common with GRK6A. These results point to GRK6A as the kinase in HEK-293 cells responsible for the high level of constitutive NHERF phosphorylation.

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